COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis

Alysia Kern Lovgren,1,2 Leigh A. Jania,3 John M. Hartney,1,3 Kelly K. Parsons,1,3 Laurent P. Audoly,4 Garret A. FitzGerald,5 Stephen L. Tilley,2 and Beverly H. Koller1,2,3

1Curriculum in Genetics and Molecular Biology, 2Department of Medicine, Division of Pulmonary and Critical Care Medicine, and 3Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; 4Department of Pharmacology, Merck Frosst Canada, Kirkland, Quebec, Canada; and 5Department of Pharmacology, University of Pennsylvania, Philadelphia, Pennsylvania

Submitted 21 November 2005; accepted in final form 8 February 2006

COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 291: L144–L156, 2006. First published February 10, 2006; doi:10.1152/ajplung.00492.2005.—Prostacyclin is one of a number of lipid mediators elaborated from the metabolism of arachidonic acid by the cyclooxygenase (COX) enzymes. This prostanoid is a potent inhibitor of platelet aggregation, and its production by endothelial cells and protective role in the vasculature are well established. In contrast, much less is known regarding the function of this prostanoid in other disease processes. We show here that COX-2-dependent production of prostacyclin plays an important role in the development of fibrotic lung disease, limiting both the development of fibrosis and the consequential alterations in lung mechanics. In stark contrast, loss of prostaglandin E2 synthesis and signaling through the Gs-coupled EP2 and EP4 receptors had no effect on the development of North Carolina, Dept. of Medicine, 4341 Molecular Biomedical Research Bldg., Chapel Hill, NC 27599 (e-mail: treawouns@aol.com).

COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 291: L144–L156, 2006. First published February 10, 2006; doi:10.1152/ajplung.00492.2005.—Prostacyclin is one of a number of lipid mediators elaborated from the metabolism of arachidonic acid by the cyclooxygenase (COX) enzymes. This prostanoid is a potent inhibitor of platelet aggregation, and its production by endothelial cells and protective role in the vasculature are well established. In contrast, much less is known regarding the function of this prostanoid in other disease processes. We show here that COX-2-dependent production of prostacyclin plays an important role in the development of fibrotic lung disease, limiting both the development of fibrosis and the consequential alterations in lung mechanics. In stark contrast, loss of prostaglandin E2 synthesis and signaling through the Gs-coupled EP2 and EP4 receptors had no effect on the development of disease. These findings suggest that prostacyclin analogs will protect against bleomycin-induced pulmonary fibrosis in COX-2−/− mice. If such protection is observed, investigation of these agents as a novel therapeutic approach to pulmonary fibrosis in humans may be warranted.

Prostaglandin; IP; lung mechanics; EP2; microsomal prostaglandin E2 synthase 1

Idiopathic pulmonary fibrosis (IPF) is a relentless, progressive, and heterogeneous disease characterized by alternating areas of normal lung, fibrosis, and interstitial inflammation affecting the peripheral subpleural parenchyma (19). Hallmarks of fibrosis include subepithelial myofibroblast/fibroblastic foci and increased deposition of collagen and extracellular matrix. This excess scar tissue causes stiffening of the alveolar walls and a decrease in compliance, which leads to the irreversible loss of total lung capacity and the reduced ability to transport oxygen into the capillaries (1, 19, 38, 47). Prostanoids, cyclooxygenase (COX)-dependent arachidonic acid metabolites, have been implicated in the development of pulmonary fibrosis. Two COX enzymes, COX-1 and COX-2, both capable of converting arachidonic acid into prostaglandin endoperoxide (PGH2), have been characterized (48). PGH2 produced by either COX isoenzyme is further metabolized by specific synthases into the various prostanooids, including prostaglandin F2 (PGF2), thromboxane, prostaglandin D2 (PGD2), prostacyclin, and PGE2. Although many cell types express both COX iso- forms and multiple synthases, colocalization of various enzymes allows the metabolite of an enzyme to be directly transferred to a specific synthase for further metabolism. Many examples of cooperation between specific COX isoforms and synthases have been described. COX-1 and thromboxane synthase couple in the production of thromboxane by platelets (41). In contrast, increasing evidence suggests that COX-2 provides PGH2 for production of prostacyclin by endothelial cells (30, 57). The pathways responsible for production of a particular prostanooid have important clinical implications. For example, the collaboration between COX-1 and thromboxane synthase in the production of thromboxane by platelets forms the basis for the use of low-dose aspirin for prevention of stroke (41). In contrast, dependence of prostanooid production by endothelial cells on COX-2 may result in loss of a critical pool of prostacyclin in patients using COX-2-specific inhibitors, leading to increased risk for cardiac events in high-risk patients (7). Another important concept regarding the COX pathway relates to changes in COX expression following cellular stimulation. Although constitutive expression of both COX isoforms has been described in certain tissues, COX-2, in contrast to COX-1, is highly inducible (48). As a result, the prostanooid profile of a resting cell can change following activation. For example, COX-1 and thromboxane synthase collaborate in production of thromboxane in resting macrophages. After activation, increased expression of microsomal PGE2 synthase 1 (mPGES1) and COX-2 parallels a drop in thromboxane synthesis (41). Microsomal PGE2 synthase 2 and cytosolic PGE2 synthase increase PGE2 production when overexpressed in various cell lines (34, 52). However, the contribution of these two enzymes to PGE2 production in vivo has not yet been defined. Similar to most eicosanoids, the half-life of PGE2 in vivo is very short due to rapid metabolism by 15-hydroxyprosta-1,5-dienoic acid hydroperoxidase (15-HPETE). The 15-HPETE products, 15-keto-PGFI2α (15-KPFI2α) and 15-keto-PGE2 (15-KPG2), can be detected by gas chromatography/mass spectrometry, which makes them useful markers for PGE2 metabolism in vivo (23). COX-2-derived prostacyclin protects against bleomycin-induced pulmonary fibrosis. Am J Physiol Lung Cell Mol Physiol 291: L144–L156, 2006. First published February 10, 2006; doi:10.1152/ajplung.00492.2005.—Prostacyclin is one of a number of lipid mediators elaborated from the metabolism of arachidonic acid by the cyclooxygenase (COX) enzymes. This prostanoid is a potent inhibitor of platelet aggregation, and its production by endothelial cells and protective role in the vasculature are well established. In contrast, much less is known regarding the function of this prostanoid in other disease processes. We show here that COX-2-dependent production of prostacyclin plays an important role in the development of fibrotic lung disease, limiting both the development of fibrosis and the consequential alterations in lung mechanics. In stark contrast, loss of prostaglandin E2 synthesis and signaling through the Gs-coupled EP2 and EP4 receptors had no effect on the development of disease. These findings suggest that prostacyclin analogs will protect against bleomycin-induced pulmonary fibrosis in COX-2−/− mice. If such protection is observed, investigation of these agents as a novel therapeutic approach to pulmonary fibrosis in humans may be warranted.

Prostaglandin; IP; lung mechanics; EP2; microsomal prostaglandin E2 synthase 1

Idiopathic pulmonary fibrosis (IPF) is a relentless, progressive, and heterogeneous disease characterized by alternating areas of normal lung, fibrosis, and interstitial inflammation affecting the peripheral subpleural parenchyma (19). Hallmarks of fibrosis include subepithelial myofibroblast/fibroblastic foci and increased deposition of collagen and extracellular matrix. This excess scar tissue causes stiffening of the alveolar walls and a decrease in compliance, which leads to the irreversible loss of total lung capacity and the reduced ability to transport oxygen into the capillaries (1, 19, 38, 47). Prostanoids, cyclooxygenase (COX)-dependent arachidonic acid metabolites, have been implicated in the development of pulmonary fibrosis.

Two COX enzymes, COX-1 and COX-2, both capable of converting arachidonic acid into prostaglandin endoperoxide (PGH2), have been characterized (48). PGH2 produced by either COX isoenzyme is further metabolized by specific synthases into the various prostanooids, including prostaglandin F2 (PGF2), thromboxane, prostaglandin D2 (PGD2), prostacyclin, and PGE2. Although many cell types express both COX iso-
taglandin dehydrogenase (PGDH). Important to this study, PGDH is expressed at high levels in the healthy lung and mice lacking this enzyme have elevated lung PGE2 levels (21).

Prostanoids mediate their actions by binding to G protein-associated receptors. Thromboxane and prostacyclin each act through a single receptor, termed TP and IP, respectively. TP is Gs coupled, and activation of this receptor leads to increased intracellular calcium levels, while activation of the Gi-coupled IP receptor leads to increased intracellular cAMP. Unlike prostacyclin and thromboxane, PGE2 binds four different E prostanoid receptors, designated EP1, EP2, EP3, and EP4 (11). These receptors have unique expression patterns and differ in their activation of intracellular signaling pathways. Activation of the EP1 receptor increases intracellular calcium (59). EP2 and EP4 are coupled to Gi and increase intracellular cAMP, although differential activation of downstream signaling components, specifically, MAPK and phosphatidylinositol 3-kinase, has been noted (43). EP3 has multiple isoforms that can couple to Gi, Go, or Gq proteins; however, Gi predominates in most systems and decreases cAMP levels (36, 51).

A number of lines of evidence suggest that PGE2 may play a role in limiting fibrotic responses in the lung and that this pathway may be compromised in patients with pulmonary fibrosis. First, patients with IPF have decreased PGE2 levels in bronchoalveolar fluid, and fibroblasts taken from these patients have decreased COX-2 expression and reduced PGE2 production (5, 58, 60). Ex vivo studies have shown that PGE2 can decrease proliferation and suppress collagen synthesis of lung fibroblasts (3, 9, 13, 14, 18, 31). Other studies have shown that PGE2 is capable of inhibiting fibroblast migration and fibroblast-to-myofibroblast transition (26, 28). Myofibroblasts express α-smooth muscle actin and have increased collagen gene expression. Thus the increased number of myofibroblasts is likely to contribute to the morphological changes as well as the contractile changes of lung parenchyma characteristic of IPF (63, 64). The ability of PGE2 to alter activity and gene expression of fibroblasts has been attributed to engagement of Gi-coupled receptors, as these actions can be mimicked by agents that elevate cAMP (8, 28). Prostacyclin, which is also capable of increasing cAMP levels in many cell types, including fibroblasts, may also have a role in the development of pulmonary fibrosis. A study on lung fibroblasts from patients with IPF demonstrated decreased levels of prostacyclin production in IPF fibroblasts vs. normal fibroblasts (12).

The studies reported here were designed to elucidate the role of prostanoids in fibrotic lung disease in vivo. Using the well-established bleomycin mouse model, we examined seven congenic mouse lines deficient in the metabolism of a prostanoid or in the ability to respond to a prostanoid. In addition to using traditional histological and biochemical methods for evaluation of disease progression, we also evaluated the impact of disease on lung mechanics. Our studies reveal an important role for the COX-2-prostacyclin synthetic pathway in limiting the fibrotic response to bleomycin.

**MATERIALS AND METHODS**

**Experimental animals.** All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by Institutional Animal Care and Use Committee guidelines of the University of North Carolina at Chapel Hill. All experiments were carried out using 8- to 12-wk-old mice unless otherwise specified. Mice lacking COX-2 (Ptgs2), COX-1 (Ptgs1), mPGES1 (Ptges), PGDH (Ptgesd), EP2 (Ptger2), EP4 (Ptger4), and IP (Ptgir) were generated as previously described (7, 10, 29, 33, 39, 53, 54). Congenic COX-1−/−, EP2−/−, and IP−/− mice were generated by backcrossing onto a C57BL/6 background for at least 12 generations. The PGDH−/− mice were backcrossed onto a C57BL/6 background for seven generations. The mPGES1−/− mice were generated on a DBA/2J genetic background and backcrossed five generations onto a C57BL/6 background. EP4−/− mice only survive on a recombinant inbred background (39). Few COX-2−/− mice survive on the 129Sv/Ev or C57BL/6 genetic background. Most die with a patent ductus arteriosus within 48 h of birth. COX-2−/− mice were generated by the intercross of 129Sv/Ev congenic COX-2−/− mice with congenic C57BL/6 COX-2+/+ animals (N8). Thus all offspring, including the COX-2−/− and control COX-2+/+ animals generated from these intercrosses, will carry maternal 129Sv/Ev alleles and paternal C57BL/6 alleles and will differ only at the COX-2 locus. Animals in different experimental groups were age- and sex-matched for each experiment. Body weights did not differ between the experimental and control mice for all genotypes. Lung mechanics were assessed first in each experimental group, and then the entire lung was removed for either histological analysis or hydroxyproline measurements.

**Bleomycin treatment.** Mice were anesthetized with 0.02 mg/kg body wt of 2,2,2-tribromoethanol and administered 50 μl of saline or bleomycin (0.05 units unless specified otherwise) diluted in saline by intratracheal instillation.

**Measurements of lung mechanics.** Mice were anesthetized 21 days after bleomycin administration with 70–90 mg/kg pentobarbital sodium (American Pharmaceutical Partners, Los Angeles, CA), tracheostomized, and mechanically ventilated at a rate of 350 breaths/min, tidal volume of 6 ml/kg, and positive end-expiratory pressure of 3–4 cmH2O with a computer-controlled small-animal ventilator (Scireq, Montreal, Canada). Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide (Baxter Healthcare, Deerfield, IL). Using custom designed software (Flexivent, Scireq), we recorded airway pressure, volume, and airflow using a precisely controlled piston during maneuvers to evaluate lung mechanics.

Pressure-volume curves were generated by a sequential delivery of seven increments of air into the lungs from resting pressure to total lung capacity followed by seven expiratory steps during which air was incrementally released. Plateau pressure was recorded when airflow returned to zero at each step. The Salazar-Knowles equation (Eq. 1) was applied to the plateau pressure measurements obtained between total lung capacity (TLC) and functional residual capacity (FRC) during the expiratory phase of the pressure-volume loop to determine static compliance (Ca) of the lung (45).

\[ V = V_{\text{max}} - Ae^{-Kp} \]

where \( V_{\text{max}} \) = volume extrapolated to infinite P, \( V \) = lung volume above FRC, A and K are constants.

Forced oscillation technique measures the impedance of the lung to an oscillatory flow of mutually prime frequencies. These impedance values are applied to a mathematical model of the lung developed by Hantos et al. (20) called the constant phase model. This model provides a means of distinguishing central airways from peripheral airways and lung parenchyma. The computer-controlled piston applies a 4-s perturbation to the lungs consisting of 13 sinusoidal components having mutually prime frequencies from 1 to 20.5 Hz with approximately hyperbolically decreasing amplitudes. Multiple linear regression is used to fit impedance spectra derived from measured pressure and volume changes to the constant phase model of the lung using Eq. 2.

\[ Z(t) = R_{\text{aw}} + i_{\text{aw}} + [G_0 - iH_o/(2\pi f t)]^a \]

where \( i = \sqrt{-1} \), \( Z(t) \) = resistance of the lung as a function of frequency, \( R_{\text{aw}} \) = a measure of central airways caliber, \( i_{\text{aw}} \) = airway inductance, \( G_0 \) = dissipative(resistive) mechanical properties of the lung tissue, \( H_o \) = conservative(elastic) mechanical properties of the lung tissue, and \( \alpha = (2/\pi)\arctan(H_o/G_0) \).
To ensure proper recruitment of all alveolar spaces, a pressure-volume curve was generated first for each animal. After this maneuver, a 4-s prime wave was performed followed by a second pressure-volume curve to obtain reported values. Each perturbation was followed by 10 s of ventilation before the next measurement was taken.

**Histology.** After assessment of lung mechanics, the lungs were inflated with 10% formalin via a tracheal cannula, removed from the thoracic cavity, and fixed overnight in formalin. Lung sections were stained with hematoxylin-eosin for routine histology and Masson’s trichrome for evaluation of collagen deposition. Histology was quantitated by digital imaging of the hematoxylin-eosin-stained sections. While blinded to genotype and treatment, an investigator captured at least five images of representative areas of each lung lobe and analyzed for septal thickening due to increased cellular infiltrate, extracellular matrix, and fibroblasts. With the software ImageJ (National Institutes of Health), the threshold was set to count the number of pixels within areas of the digital images that had increased inflammation and extracellular matrix deposition. The threshold number of pixels was then divided by the total number of pixels in the entire image and multiplied by 100 to generate a percentage of area affected by fibrosis and inflammation (2).

**Hydroxyproline assay.** After assessment of lung mechanics, mice were exsanguinated, and the lungs were perfused with sterile PBS and removed. Tissue samples were homogenized in 5 ml of PBS and sonicated. Aliquots (500 µl) of each sample were lyophilized for 24 h until dry and then hydrolyzed in 500 µl of 6 N hydrochloric acid at 120°C overnight. Samples were then dried in a speed vacuum for 2 h, resuspended in water, applied to a filter Eppendorf tube (Fisher Scientific), and spun at maximum speed for 5 min. Aliquots were assayed by adding chloramines-T for 20 min at room temperature and then developing them with Ehrlich-perchloric acid at 65°C for 15 min. Absorbance was read at 561 nm in a spectrophotometer, and samples were compared with a standard curve generated from known concentrations of hydroxyproline standard (Sigma) (23).

**PGE2 analysis.** Mice were killed 7 days postbleomycin instillation with pentobarbital sodium and exsanguinated. Lungs were removed and flash frozen in liquid nitrogen. Samples were homogenized in 1× PBS with 1 mM EDTA, pH 7.4, and 10 mM indomethacin and sonicated. Lipids were purified through a C18 column, and PGE2 content was determined by using enzyme immunoassay kits (Assay Designs).

**Statistical analysis.** Values are presented as means ± SE and are analyzed by Student’s two-tailed t-test or ANOVA followed by Tukey-Kramer honestly significant difference post hoc test. A P value <0.05 is considered statistically significant.

**RESULTS**

**Contribution of COX-1- and COX-2-derived prostanoids to bleomycin-induced pulmonary fibrosis.** A number of early studies using mice lacking COX-2 suggested that COX-2-derived prostanoids could limit some aspects of the histological changes and increases in collagen production observed in the bleomycin and vanadium pentoxide (V2O5) models of interstitial fibrosis (4, 25). However, in a recent study, this difference in disease progression in the COX-2−/− mice was not as apparent (22). A possible explanation for inconsistent findings is suggested on examination of genetic background of the COX-2 mice used in the various studies. To address this potential concern, we examined the role of COX-2 in the bleomycin model of fibrosis using congenic F1 animals. In addition to using traditional histological and biochemical methods for evaluating disease progression in the COX-2−/− mice, we assessed the impact of loss of COX-2 on changes in airway mechanics characteristic of pulmonary fibrosis.

Bleomycin or saline was administered to cohorts of sex-matched COX-2−/− mice and wild-type littermates. Lungs were harvested 21 days after treatment, and histological changes were assessed by analysis of hematoxylin-eosin and Masson’s trichrome-stained sections of lung. As expected, no histological changes were observed in the lungs from the saline-treated animals (Fig. 1, A and B). As previously described for this model, remarkable structural alterations including increased collagen deposition and cellularity were observed in lungs from animals exposed to bleomycin. These histological changes appeared more pronounced in the COX-2−/− animals (Fig. 1, C–F). A digital imaging program was used to quantify the extent of fibrosis and inflammation in the lungs as described in the materials and methods (2). Figure 1G demonstrates increased disease progression in lungs of the COX-2−/− mice compared with the congenic controls. The extent of collagen deposition was assessed biochemically by quantitating hydroxyproline levels in lung homogenates from similarly treated cohorts of COX-2−/− and COX-2+/+ animals. Consistent with qualitative differences observed on examination of trichrome-stained sections, COX-2−/− mice had increased levels of hydroxyproline after bleomycin administration compared with wild-type mice (Table 1).

In humans, interstitial fibrosis leads to alterations in lung mechanics characterized by a decrease in lung compliance. We therefore determined whether similar changes in airway mechanics could be observed in the bleomycin model of pulmonary fibrosis and, furthermore, whether these changes were sensitive to the presence or absence of COX-2-derived prostanoids. For this analysis, we utilized a computer-controlled small-animal ventilator, highly sensitive pressure transducers, and software (Flexivent) to record airway opening pressures, volume, and airflow. Changes in lung mechanics were determined using two different methods. Static compliance was determined from analysis of pressure-volume curves, and tissue elastance was measured by applying the constant phase model to prime wave impedance values obtained using the forced oscillation technique. Decreases in compliance and increases in elastance are anticipated in the bleomycin-exposed lung if this treatment indeed models pulmonary fibrosis. Consistent with this expectation, wild-type bleomycin-injured mice showed a significant decrease in static compliance and a significant increase in tissue elastance compared with saline-treated mice (P < 0.005). The constant phase model of the lung also provides information concerning two additional parameters, airway resistance (Raw) and tissue resistance (G). Consistent with the sparing of the conducting airways in this model, no change in Raw was observed in the mice after bleomycin treatment. A small, but significant, increase in G was observed in all animals treated with bleomycin; however, this parameter did not allow us to distinguish between mice with differences in disease severity.

Sex-matched COX-2−/− mice and their littermate controls were administered either bleomycin or saline, and lung mechanics were assessed 21 days later. Both groups had a significant decrease in static compliance after bleomycin administration compared with the saline controls. However, the static compliance of the bleomycin-treated COX-2−/− mice was significantly lower than the static compliance of the bleomycin-treated COX-2+/+ mice (Fig. 2A). Accordingly, a significantly higher tissue elastance was measured in the COX-2−/−
mice compared with COX-2−/− mice after bleomycin administration (Fig. 2B). The significant increase in tissue elastance and significant decrease in static compliance along with the histological and biochemical differences suggest that COX-2-derived prostanoid(s) protect against the development of bleomycin-induced pulmonary fibrosis.

In addition to COX-2, the lung expresses high levels of COX-1. We next determined whether a similar role for COX-1-derived prostanoids could be observed in this model. As COX-1−/− mice survive on the C57BL/6 genetic background, C57BL/6 COX-1−/− animals and their congenic controls were used for these studies. Unlike the COX-2−/− mice, no discernable difference in disease pathogenesis was observed between the COX-1−/− animals and their controls in any of the disease parameters. Table 1 shows a similar increase in hydroxyproline content in the COX-1−/− and COX-1+/+ mice after bleomycin administration. Consistent with this, both histological analysis and measurements of lung mechanics demonstrate no differ-

Fig. 1. Histological analysis reveals increased cellularity and deposition of collagen in the lungs of cyclooxygenase (COX)-2−/− mice compared with wild-type mice after bleomycin (Bleo) instillation. Lungs were harvested 21 days following treatment and fixed overnight in formalin. Lung sections were stained with hematoxylin-eosin or Masson’s trichrome. Original magnification, ×10. A: saline-treated wild-type mouse, hematoxylin-eosin stain. B: saline-treated COX-2−/− mouse, hematoxylin-eosin stain. C: Bleo-treated wild-type mouse, hematoxylin-eosin stain. D: Bleo-treated COX-2−/− mouse, hematoxylin-eosin stain. E: Bleo-treated wild-type mouse, Masson’s trichrome stain. F: bleomycin-treated COX-2−/− mouse, Masson’s trichrome stain. G: morphological changes observed in lungs obtained from bleomycin-treated mice were quantitated by digital imaging of hematoxylin-eosin-stained lung sections and presented as a percentage of septal thickening. Saline-treated COX-2+/+, n = 9; saline-treated COX-2−/−, n = 6; Bleo-treated COX-2+/+, n = 12; Bleo-treated COX-2−/−, n = 11. *P < 0.01 compared with corresponding saline value, #P < 0.01 compared with COX-2−/− Bleo value.
Table 1. Changes in hydroxyproline content

<table>
<thead>
<tr>
<th></th>
<th>+/+</th>
<th>−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>COX-2</td>
<td>50.85 ± 10.86</td>
<td>125.12 ± 27.94*</td>
</tr>
<tr>
<td>COX-1</td>
<td>64.75 ± 19.82</td>
<td>75.95 ± 22.55</td>
</tr>
<tr>
<td>EP2</td>
<td>78.77 ± 25.29</td>
<td>56.46 ± 20.93</td>
</tr>
<tr>
<td>EP4</td>
<td>212.78 ± 19.46</td>
<td>193.50 ± 17.40</td>
</tr>
<tr>
<td>IP</td>
<td>51.22 ± 8.81</td>
<td>105.48 ± 18.03*</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with corresponding wild-type value; n = 8 – 11. Lungs were removed for hydroxyproline analysis 21 days after treatment. Mean saline treated values were subtracted from the bleomycin-treated values to obtain the change in hydroxyproline content in µg/lung. Values presented are means ± SE in µg/lung.

ence in disease progression between the two groups (Supplemental Fig. S1; supplemental data for this article may be found at http://ajplung.physiology.org/cgi/content/full/00492.2005/DC1). Thus development of fibrotic lung disease after exposure to bleomycin is modulated by COX-2-, but not COX-1-, dependent prostanoid production.

Alterations in PGE2 levels fail to alter susceptibility to bleomycin-induced pulmonary fibrosis. COX-2-derived PGH2 can be further metabolized into prostacyclin, thromboxane, and the prostaglandins PGE2, PGD2, and PGF2. Both anti-inflammatory and antifibrotic properties have been attributed to PGE2, which suggests that this prostanoid, acting through either the EP2 or EP4 Gs-coupled receptor, can limit progression of fibrotic lung disease. To test this hypothesis, we examined the development of lung disease after bleomycin treatment in mice lacking mPGES1, the inducible PGE2 synthase.

Three enzymes have been reported to metabolize PGH2 into PGE2 in vitro; however, only one of these, mPGES1, has been demonstrated to contribute to PGE2 production in vivo. We first established the contribution of mPGES1 to the increases in PGE2 levels in the lungs observed after exposure to bleomycin. Mice lacking mPGES1 and congenic control animals were treated with bleomycin or saline. PGE2 levels were measured in whole lung homogenates 7 days after treatment. As expected, significant increases in lung PGE2 levels were observed in wild-type mice following bleomycin treatment. In contrast, increases in lung PGE2 were severely attenuated in mPGES1−/− mice (Fig. 3). Thus the marked increase in PGE2 levels after bleomycin administration is almost completely dependent on mPGES1 expression.

We next determined the impact of this relative PGE2 deficiency on the pathogenesis of bleomycin-induced fibrotic lung disease. C57BL/6 mPGES1+/− mice and congenic controls were administered bleomycin or saline, and histological changes in the lung were evaluated 21 days after treatment. Surprisingly, no difference could be discerned in development of disease in the two groups. Quantitation of inflammation and extracellular matrix deposition by digital analysis demonstrated a similar extent and severity of interstitial inflammation and fibrosis (Fig. 4A). We further evaluated disease progression in the mPGES1−/− mice by measuring changes in lung function following bleomycin administration. Both loss of compliance and increase in elastance as a result of bleomycin exposure were of similar magnitude in mPGES1−/− vs. mPGES1+/+ mice (Fig. 4, B and C).

We next considered the possibility that an increase in disease severity resulting from decreased PGE2 production may be difficult to observe in these experiments because of the extensive disease induced even in the wild-type controls. To address

Fig. 2. Analysis of lung mechanics demonstrates increased disease susceptibility in the COX-2−/− mice. Lung mechanics were measured in anesthetized, paralyzed, and mechanically ventilated mice 21 days following Bleo or saline instillation. A: static compliance (Cst) determined by fitting the Salazar-Knowles equation to pressure-volume curves; n = 6–9 animals; *P < 0.05 compared with corresponding saline value, #P < 0.05 compared with COX-2+/+ Bleo value. B: tissue elastance (H) determined by applying prime wave impedance values to the constant phase model; n = 9–11 animals; *P < 0.05 compared with corresponding saline value, #P < 0.05 compared with COX-2+/+ Bleo value.

Fig. 3. Reduced prostaglandin E2 (PGE2) levels in the lung homogenates of microsomal prostaglandin E2 synthase 1 (mPGES1)−/− mice compared with wild-type mice after Bleo administration. The lungs were harvested 7 days after Bleo or saline administration. n = 3; *P < 0.01 compared with corresponding saline value, #P < 0.01 compared with mPGES1+/+ Bleo value.
elastance after both high- and low-dose bleomycin administration compared with the saline-treated controls (Fig. 4, B and C). Thus loss of mPGES1-dependent PGE2 production does not cause enhanced susceptibility to bleomycin-induced fibrosis.

To further address the role of PGE2 in bleomycin-induced pulmonary fibrosis, we examined mice lacking PGDH, the major catabolic enzyme for PGE2. Previous studies in our laboratory have shown that these mice have increased lung PGE2 levels (21). If PGE2 can limit the initiation or progression of fibrosis, we anticipated that the loss of PGDH and consequential increased PGE2 levels might protect these mice from developing pulmonary fibrosis. However, both pathological and physiological measurements revealed no difference in disease progression between the PGDH−/− and PGDH+/+ mice after bleomycin administration (Supplemental Fig. S2).

Loss of PGE2 Gα-coupled receptors does not enhance susceptibility to bleomycin-induced fibrosis. Although the majority of PGE2 production in the bleomycin-treated mouse lung is dependent on mPGES1, it is possible that it is the loss of local discrete pools of mPGES1-independent PGE2 that plays a critical role in limiting the extent of fibrosis within the lung parenchyma. To address this possibility, mice lacking the receptors through which PGE2 activates fibroblasts and other cell types important to disease pathogenesis were examined. We anticipated that the Gα-coupled EP2 and EP4 receptors, rather than the Gαq/q-coupled EP3 receptor or the Gq-coupled EP1 receptor, would be more likely to mediate the protective actions of PGE2 in the lung, so we examined the development of disease in the EP2−/− mice first. Quantitative analysis of the histological changes revealed no significant differences between the EP2−/− mice and wild-type mice in the extent and severity of interstitial inflammation and fibrosis after bleomycin administration (Fig. 5A). Consistent with these findings, hydroxyproline analysis revealed similar increases in collagen deposition after bleomycin administration in the wild-type and EP2−/− mice (Table 1). We further analyzed disease progression in the EP2−/− mice by assessing alterations in lung mechanics. Both EP2−/− and wild-type mice had a similar decrease in static compliance and increase in tissue elastance after bleomycin administration (Fig. 5, B and C). A similar result was obtained following treatment of 4- to 6-mo-old EP2−/− and wild-type animals with 0.1 unit of bleomycin (Fig. 5, D and E).

Because EP4−/− mice only survive on a mixed genetic background, a recombinant inbred (RI) background permissive to this mutation was generated (39). Congenic animals have been produced by the successive intercross of −/− and +/+ animals for 20 generations, after which EP4−/− and control animals were generated. This RI strain is sensitive to bleomycin-induced lung injury, with treated mice showing both histological changes and alterations in lung function characteristic of this model. In fact, exposure to bleomycin induces a more robust increase in collagen deposition in the RI mouse strain than is observed in either C57BL/6 or the F1 (C57BL/6 and 129/SvEv) mice. The RI strain also displays a lower basal lung compliance than other mouse strains. Age- and sex-matched EP4−/− mice and their congenic controls were administered either bleomycin or saline, and lung mechanics were assessed 21 days later. A similar decrease in static compliance and increase in tissue elastance was observed in both EP4−/− and

This concern, we repeated our comparison of the mPGES1−/− and control animals inducing a milder disease by decreasing the dose of bleomycin (0.025 units rather than 0.05 units). As expected, the severity of the fibrotic response was reduced in the wild-type mice. However, no difference could be discerned between the mPGES1−/− and mPGES1+/+ mice. Both the mPGES1−/− and mPGES1+/+ mice had a significant but similar decrease in static compliance and increase in tissue
control animals after exposure to bleomycin (Fig. 6). Consistent with these physiological observations, hydroxyproline levels were similar between the EP4−/− mice and the EP4+/+ controls after bleomycin administration (Table 1). Together, these studies with mice lacking the Gs-coupled PGE2 receptors and mice lacking the dominant enzyme responsible for PGE2 synthesis in the lung suggest that a COX-2-derived prostanoid other than PGE2 is responsible for limiting lung fibrosis in this model.

IP−/− mice are more susceptible to bleomycin-induced fibrosis. The failure to observe increased disease in mice deficient in PGE2 production, together with a recent collaborative study showing increased fibrotic lesions in the hearts of mice lacking the IP receptor (16), suggested that COX-2-dependent production of prostacyclin, and not PGE2, may be critical in limiting development of fibrotic lung disease. To test this hypothesis, congeneric C57BL/6 IP−/− and wild-type mice were administered bleomycin or saline, and disease was assessed 21 days later by histological analysis, hydroxyproline measurements, and lung mechanics. As expected, no difference was observed between the histology of the saline-treated IP−/− and wild-type mice (Fig. 7, A and B). Interestingly, and similar to the COX-2−/− mice, the IP−/− mice had more severe disease demonstrated by a significant increase in all disease parameters. Histological analysis of the IP−/− mice demonstrated increased collagen deposition and cellularity after bleomycin administration compared with the wild-type mice (Fig. 7, C–F). This correlated with a significant increase in the quan-
Bleomycin-induced fibrosis is extensively used to model aspects of the pathogenesis of interstitial pulmonary fibrosis. Here we show that intratracheally administered bleomycin leads to changes in lung mechanics that parallel the observed in patients with pulmonary fibrosis, providing an additional means of assessing the severity of disease in the mouse. We examined the development of fibrotic lung disease in seven congenic mouse lines, each carrying a null allele in a gene required for the normal synthesis or response to prostanoids. These studies show that COX-2-dependent prostacyclin production limits the development of fibrosis and subsequent loss of lung function after exposure to bleomycin. In contrast, alterations in PGE2 levels or in the ability of mice to respond to PGE2 have no effect on the development of disease.

Pulmonary function testing is commonly used to monitor the progression of pulmonary fibrosis in humans. Reductions in forced vital capacity reflect changes in lung compliance as collagen is synthesize by myofibroblasts and deposited within the interstitium of the lung parenchyma. Similar measurements have not typically been utilized in evaluation of disease in this mouse model of interstitial pulmonary fibrosis. Rather, the severity of disease induced by bleomycin treatment is assessed by semiquantitative histological examination and changes in lung hydroxyproline content. In our studies, in addition to these traditional criteria, we measured lung mechanics of both saline- and bleomycin-exposed animals. In all experiments, treatment of both wild-type and congenic lines with bleomycin results in a significant decrease in static compliance, determined by analysis of pressure-volume loops from tracheostomized, mechanically ventilated animals. In addition to detecting differences between control and bleomycin-treated animals, assessment of static compliance allows us to distinguish differences in disease severity in various experimental groups. The increase in fibrotic disease in the bleomycin-treated COX-2−/− and IP−/− animals detected by histological and biochemical measurements was paralleled by a significant decrease in static compliance of the lungs. In the other mice tested, when histological evaluation and hydroxyproline measurements failed to distinguish between the two experimental groups of mice, no difference could be observed in the static lung compliance of these animals.

We also evaluated the changes in lung mechanics after bleomycin exposure using the forced oscillatory technique. This method, pioneered by Schuessler, Bates, and Irvin (24, 46), measures the impedance of the lung to an oscillatory flow of mutually prime frequencies. The constant phase model of the lung developed by Hantos et al. (20) is then utilized to calculate a value termed tissue elastance, which reflects the conservative (elastic) mechanical properties of the lung tissue. Tissue elastance was increased in all mice exposed to bleomycin. Experimental groups with more severe disease, on the basis of histological and biochemical parameters, also displayed a significant increase in tissue elastance. Thus both tissue elastance and static compliance provide a sensitive means of assessing changes in lung mechanics that parallel the morphological changes induced by exposure to bleomycin.

Our observation of increased disease in the COX-2−/− bleomycin-treated mice is consistent with the report by Keerthisingam et al. (25) using this same model of fibrotic lung disease. In their study, histological examination of a small group of mice of mixed genetic background revealed more severe disease in the COX-2−/− animals. However, in a more recent report by these investigators, the degree of fibrosis in the lungs was similar between bleomycin-treated COX-2−/− mice and similarly treated wild-type controls (22). Mice used in these studies were generated from selection and breeding of populations of F2 mice carrying both 129Sv/Ev and C57BL/6 genes, mice much less susceptible to patent ductus arteriosus.
Over time, this breeding scheme likely results in a skewing of the genetic background of the mice in favor of a particular complement of 129Sv/Ev and C57BL/6 alleles. Differences in the response of the COX-2+/- animals used in the various experiments could be attributed at least in part to distorted representation of 129Sv/Ev and C57BL/6 alleles in control and COX-2+/- animals. Bonner et al. (4) examined COX-1+/- and COX-2+/- mice of mixed genetic background using a V2O5 model of pulmonary fibrosis. V2O5 exposure does not result in fibrotic disease in wild-type mice. Although loss of COX-1 did not result in an increase in collagen deposition, a measurable increase in fibrosis was observed in the COX-2+/- animals. These results correlate well with our studies of the C57BL/6 congenic COX-1+/- mice and the F1 COX-2+/- mice in the bleomycin model. We also failed to observe a difference in the development of disease in mice lacking COX-1 expression,
and, on the other hand, we found that the fibrotic response to bleomycin is exaggerated in the COX-2−/− mice. Together, histological and biochemical studies of the COX-deficient mice in both the bleomycin and V2O5 models of pulmonary fibrosis and analysis of lung mechanics after exposure to bleomycin suggest a role for COX-2-derived prostanoids in limiting fibrotic lung disease.

A number of lines of evidence indicate that the protective prostanooid produced by the COX-2 pathway is PGE2 and that the protective actions of PGE2 are mediated by the EP2 receptor. This model, however, is not supported by the studies reported here. First, we found no increase in disease parameters in the mice lacking mPGES1, despite our finding that this synthase is responsible for the increase in PGE2 levels measured in the mouse lung after exposure to bleomycin. Two other proteins capable of metabolizing PGH2 to PGE2 have been identified, although a role for these proteins in the in vivo production of PGE2 has not yet been reported. However, as the phenotype of the mPGES1−/− does not recapitulate the phenotypes of the various receptor deficient mice, it is presumed that some mPGES1-independent pathways must be active in vivo (39, 53). It is possible that these alternative synthetic pathways provide discrete pools of PGE2 essential for protecting mice against bleomycin-induced disease. We therefore examined mice lacking PGE2 receptors, focusing on the two

Gαi-coupled PGE2 receptors, EP2 and EP4. No difference in the response of the mice to bleomycin was observed in either the EP2−/− or EP4−/− mice compared with controls. Our results differ from a recent study carried out using EP2−/− mice. In this study, modest increases in collagen levels were measured in the EP2−/− animals compared with controls (32). The basis for these conflicting results is not apparent. However, our inability to observe changes physiologically, biochemically, and pathologically in mPGES1−/−, PGDH−/−, EP2−/−, and EP4−/− mice does not support an antifibrotic role for PGE2 in this model.

Prostacyclin production, similar to PGE2, is dependent on COX metabolism of arachidonic acid. Prostacyclin binds with high affinity to the IP receptor, a seven-transmembrane receptor that, like the EP2 and EP4 receptors, couples primarily to Gs to activate adenylyl cyclase. IP receptors are expressed at high levels on platelets, and prostacyclin limits platelet aggregation and thrombi formation. Loss of COX-2-mediated prostacyclin production by endothelial cells and the resultant unopposed activity of COX-1-dependent thromboxane action on platelets may underlie possible increased risks for cardiovascular events in patients treated with COX-2-specific inhibitors (15). Fewer studies have examined the role of prostacyclin in fibrotic lung diseases such as IPF.

A role for prostacyclin in pulmonary fibrosis is consistent with the expression of prostacyclin synthase (PGIS) and the IP receptor by many cell types present in the lung. PGIS expression has been demonstrated on pneumocytes, fibroblasts, endothelial cells, and resident leukocytes, and IP expression has been demonstrated on pneumocytes, fibroblasts, smooth muscle cells, and macrophages (12, 27, 40, 42, 44, 55). In addition, IP receptors are expressed by many hematopoietic cells, including lymphocytes and neutrophils, recruited to the lung after exposure to bleomycin (37, 61). We have examined the expression of PGIS and IP by quantitative RT-PCR in salinetreated and bleomycin-treated lungs. As expected, expression of both the synthase and the receptor was easily detected in the healthy lung. PGIS expression was not increased after induction of fibrosis with bleomycin. However, a small (50%) increase in the expression of the IP receptor was observed using this method (Supplemental Fig. S3).

We recently reported that mice lacking IP receptors have elevated blood pressure, and the hearts of these animals showed extensive fibrosis (16). Interestingly, while fibrosis is an expected consequence of elevations in blood pressure and cardiac stress, the extent of fibrosis in the IP-deficient mice was exaggerated compared with the modest increase in blood pressure. This suggests that, while the fibrosis was likely triggered by the stress conferred by elevated blood pressure, the fibrotic response normally may be limited by the presence of IP receptors on cardiac fibroblasts. Consistent with this interpretation, stable prostacyclin analogs can inhibit migration of lung fibroblasts and proliferation of cardiac fibroblasts (27, 62). Exposure to IP agonists also decreased expression of types I and III collagen by cardiac fibroblasts (62). In fact, bradykinin-mediated decreases in collagen formation by these cells are the indirect consequence of production of a prostanoid, particularly prostacyclin, the major prostanooid formed by cardiac fibroblasts (17).

Studies of dermal fibroblasts further support a role for prostacyclin in regulation of fibroblast growth and production

![Graph](http://ajplung.physiology.org/)

Fig. 8. Analysis of lung mechanics demonstrates increased disease susceptibility in the IP−/− mice. Lung mechanics were measured in anesthetized, paralyzed, and mechanically ventilated mice 21 days following Bleo or saline instillation. A: Cst determined by fitting the Salazar-Knowles equation to pressure-volume curves. B: H determined by applying prime wave impedance values to the constant phase model. Saline-treated, n = 8–10; Bleo-treated, n = 15–18. *P < 0.05 compared with corresponding saline value. #P < 0.05 compared with IP−/− Bleo value.
of extracellular matrix components. The prostacyclin analog, iloprost, suppressed type I collagen induction by transforming growth factor (TGF)-β in dermal wounds and attenuated induction of connective tissue growth factor (CTGF) (49). Iloprost was also shown to suppress CTGF in the dermis of sclerodema patients (50). Further studies with dermal fibroblasts suggest a mechanism whereby stimulation of the IP receptor leads to PKA activation, which in turn limits TGF-β induction of collagen and CTGF by suppressing the Ras/MEK/ERK cascade (49). Together, these studies support a model in which induction of COX-2 in the bleomycin-treated mice leads to increased production of prostacyclin. Prostacyclin acts on IP receptors expressed by fibroblasts to limit their response to injury, including their migration to the lung, proliferation, and production of collagen.

We cannot yet rule out the possibility that the increase in disease observed in the IP−/− mice is not the consequence of loss of the inhibitory actions of prostacyclin on the fibrotic response itself but, rather, the result of an enhanced inflammatory response following bleomycin treatment. IP receptors are expressed on many leukocyte populations, and an increase in cAMP in these cells would be expected to limit migration and production of proinflammatory cytokines. In fact, the studies with the COX-2 mice in the V2O5 model support this interpretation (4). Future studies in which loss of the IP receptor is limited to specific populations should allow us to further define the mechanism by which COX-2-dependent prostacyclin production limits the development of fibrosis in the mouse lung.

In these studies, we show that loss of the prostacyclin receptor has a profound impact on the development of bleomycin-induced pulmonary fibrosis. IP−/− mice showed increased disease by all parameters examined. The magnitude of the physiological, biochemical, and pathological changes was similar to that of COX-2−/− mice, suggesting that loss of prostacyclin, and not loss of PGE2, is responsible for the enhanced fibrotic response observed in our studies. The importance of COX-2-dependent prostacyclin production in limiting thrombotic events by counteracting the prothrombotic actions of thromboxane has been extensively explored (7). The studies reported here suggest that COX-2-dependent prostacyclin production may also have an important protective role in fibrotic diseases. In this regard, Murakami et al. (35) recently examined the impact of a prostacyclin agonist on the development of fibrosis in the bleomycin model. Interpretation of their findings, however, is complicated by the fact that this compound is not similar to that of COX-2−/− mice, suggesting that loss of COX-2 and prostacyclin, and not loss of PGE2, is responsible for the enhanced fibrotic response observed in our studies. The importance of COX-2-dependent prostacyclin production in limiting thrombotic events by counteracting the prothrombotic actions of thromboxane has been extensively explored (7). The studies reported here suggest that COX-2-dependent prostacyclin production may also have an important protective role in fibrotic diseases. In this regard, Murakami et al. (35) recently examined the impact of a prostacyclin agonist on the development of fibrosis in the bleomycin model. Interpretation of their findings, however, is complicated by the fact that this compound is not similar to that of COX-2−/− mice, suggesting that loss of COX-2 and prostacyclin, and not loss of PGE2, is responsible for the enhanced fibrotic response observed in our studies.

REFERENCES


